Letters in Applied Microbiology 2005, **41,** 428–433 doi:10.1111/j.1472-765X.2005.01761.x

Modulation of water activity on fungicide effect on *Aspergillus niger* growth in Sabouraud dextrose agar medium*

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2004/1453: received 15 December 2004, revised 26 January 2005 and accepted 3 May 2005

ABSTRACT

X. NI AND D.A. STREETT. 2005.

Aims: To examine whether water activity (a_w) in combination with low concentration of fungicides can be used to effectively control *Aspergillus niger* van Tieghem growth in cultural medium, the Sabouraud dextrose agar (SDA). The data would be used as baseline information for reducing *A. niger* contamination in insect artificial diets.

Methods and Results: *Aspergillus niger* was isolated from an insect artificial diet. Four concentration levels (i.e. 0, 1, 10 and 20 μmol) of two fungicides (i.e. amphotericin B and itraconazole) were tested against *A. niger* under four a_w levels (i.e. 0·994, 0·961, 0·921 and 0·859) adjusted by including 0, 12·5, 25 and 38% of glycerol in the medium mixture. *Aspergillus niger* growth was significantly reduced at low fungicide concentration (1 μmol), and at reduced a_w. The spore germination was prevented with either higher fungicide concentration (>10 μmol), or low a_w in the medium ($a_w < 0{\cdot}921$). The two ecological determinants (fungicides and a_w) showed a significant impact on *A. niger* survival in the medium ($P < 0{\cdot}0001$). Itraconazole is more effective than amphotericin B in controlling *A. niger* contamination in the agar medium.

Conclusion: Adjustment of a_w (with 12·5% of glycerol) in combination with 1 μmol of itraconazole can effectively prevent *A. niger* growth in the SDA cultural medium.

Significance and Impact of the Study: *Aspergillus niger* contaminations have frequently affected the quality of insects produced from mass rearing facilities. Low a_w in combination with low fungicide concentration has the potential to become one of the most cost-effective management strategies to prevent *A. niger* contamination in insect artificial diets. The effect of fungicides and low a_w in artificial diets on insect biology needs to be further examined.

Keywords: amphotericin B, colony diameter, filamentous fungus, germ tube extension, glycerol, itraconazole, spore germination.

INTRODUCTION

Fungal contamination has been and continues to be an important spoilage factor of artificial diets for mass rearing of insects, which inflicts periodical disruptions of mass production and continuous supply of insects for commercial and research uses. Both environmental and dietary factors are equally important for reducing diet spoilage and improving the efficacy of mass production. Besides improved sanitation of the production process, the adjustment of the ecological factors in an insect diet for mass rearing could be an effective measure to reduce the contamination of *Aspergillus niger* van Tieghem in insect rearing. Although *A. niger* contamination of insect diets has been studied for decades (Clark *et al.* 1961; Gifawesen *et al.* 1975), fungal and microbial spoilage of insect diets in general is still a serious periodic problem in insect mass rearing facilities (Funke 1983; Sikorowski and Lawrence 1994, 1997). In particular, *A. niger* is commonly known as an insidious rearing facility contaminant, as well as a pathogen for young

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lepidopteran larvae in mass rearing facilities (Sikorowski and Lawrence 1997).

A number of recent studies have examined a variety of intrinsic (e.g. pH, moisture content) and extrinsic (e.g. temperature, relative humidity) properties of food and animal feed to reduce microbial contamination (Jay 2000; Marin *et al.* 2003). Alverson and Cohen (2002) and Alverson (2003) demonstrated that benzoic and sorbic acids could be used as effective antifungal agents for insect diets without compromising insect biological fitness. Nesci *et al.* (2003) examined the impact of different water activity (a_w) and pH conditions on the efficacy of using antioxidants to suppress *Aspergillus* spp. and aflatoxin production on maize. They demonstrated that butylated hydroxyanisole, and propyl paraben were effective for controlling *Aspergillus flavus* and *Aspergillus parasiticus* growth and aflatoxin production in stored maize. Low a_w significantly reduced spore (*Aspergillus* spp.) germination. The spores only germinated on the medium with high a_w values (i.e. 0·982 and 0·937), while the spores did not germinate when a_w values were 0·809 and 0·747. In comparison with a_w values, variable pH values (i.e. 6, 7 and 8) had much less effect on the growth of *Aspergillus* spp. (Nesci *et al.* 2003).

The objectives of this study were to assess the effects of low water activity (a_w) in combination with low concentration of fungicides on suppressing *A. niger* in a common culture medium – Sabouraud dextrose Agar (SDA). This is the initial study of a series of experiments to evaluate the effectiveness of adjusting ecological determinants to reduce *A. niger* contamination of insect diets in mass production of *Chrysoperla* and other insects.

MATERIALS AND METHODS

Fungal culture

The fungal culture used in this study was isolated from the Gast Rearing Facility (Mississippi State, MS) and an insect diet. Thus, the culture represents the contaminating organism found in the insect diets at the facility. The culture was identified as *A. niger* van Tieghem according to St-Germain and Summerbell (1996) and Hocking (1997).

Culture medium preparation

Sabouraud dextrose agar (pH = 5·6) (ICN Biomedicals, Inc., Costa Mesa, CA, USA) was used in this study. The water activity of the medium could be adjusted at 0·994, 0·950, 0·900 and 0·850 by adding glycerol (Marin *et al.* 1995a,b, 1998). While Nesci *et al.* (1998) reported that *A. niger* spores did not germinate when $a_w = 0{\cdot}800$, Jay (2000) stated that most fresh food a_w value is above 0·99, which is similar to the a_w values of insect diets. In the Gast insect-rearing facility, the a_w value for lepidopteran insect diet is 0·999, and the NI diet for *Lygus* spp. is 0·998 (Cohen 2004).

In a preliminary study, we determined using a standard curve that 0, 12·5, 25 and 38% of glycerol used to replace the same amount of water needed in the SDA medium would make the medium to have a_w values of 0·994, 0·961, 0921 and 0·859. Each of these a_w values was the average of six readings that recoded during the experiment using a water activity meter (Model CX-2, Aqua Lab, Decogan, Pullman, WA, USA). The four levels of a_w were selected to cover a_w values of a variety of insect diets as well as the optimum a_w value for preventing *A. niger* contamination according to previous report by Jay (2000) and Nesci *et al.* (2003). The SDA medium with different amount of glycerol was autoclaved at 121°C for 15 min, then cooled to approx. 45–50°C, added the fungicides, and then poured into 9-cm sterile Petri dishes with quadrant sections (Fisher Scientific, St Louis, MO, USA).

Antifungal agents

Chemical food preservatives and antimicrobial agents (e.g. sodium benzoate, sorbic acid and tetracycline) have been widely used in the artificial diets for the mass rearing of a number of insect species (Sikorowski and Lawrence 1994). Antifungal agents used here were selected according to previous reports on the control of *Aspergillus* spp. by Espinel-Ingroff *et al.* (1997) and Provine and Hadley (2000). Amphotericin B was initially dissolved in dimethyl sulfoxide, while itraconazole was dissolved in 0·5 ml of chloroform. The dissolved chemicals were then diluted into 5 ml of stock solution in 95% ethyl alcohol. The concentrations for each fungicide were 0, 1, 10 and 20 μmol at each of the four a_w levels.

Spore germination

The germination test protocol was adopted from Nesci *et al.* (2003) with modification. Briefly, after *A. niger* was cultured on the SDA medium at 27°C for 5 days, the conidia from the culture were collected and suspended in 1 ml of sterile water. The spore concentration in the suspension was determined using a haemocytometer. The experiment was initiated by adding 2 μl of spore suspension (with $8{\cdot}2 \times 10^6$ spores per ml) onto the solidified agar plates, which had been amended with appropriate concentrations of the antifungal agents. The plates with the same water activity treatments were placed and sealed in a polyethylene bag during the incubation at 27°C. The experiments were repeated three times. The spore was considered germinated when the germ tube was longer than the diameter of the spores (Nesci *et al.* 2003). Three indices were measured:

(i) the percentage of spore germination was recorded at 7, 12, 17, 22, 48, 72, 96 and 168 h after the inoculation by randomly selecting and examining 100 spores in each replication; (ii) the lag-phase of spore germination was determined using the number of the hours needed for 10% spore germination; and (iii) the extension of germ tube was measured at different time intervals and calculated and expressed as μm per hour after randomly selecting measuring 10 germ tubes for each replication.

Fungal growth

The growth rate of *A. niger* on the SDA plates cultured at 27°C was assessed according to colony diameter measurements. The diameter of each colony was determined using two measurements recorded at right angles to one another. The colony growth rate (mm day^{-1}) was then calculated using the difference between the two diameter measurements divided by the number of days between them.

Experimental design and data analysis

This experiment utilized a randomized complete block design with repeated measures, which is also known as a special type of split-plot design (Neter *et al.* 1985). The experiment was repeated three times. Each experiment had four levels of water activity ($a_w = 0{\cdot}994$, 0·961, 0·921 and 0·859) and two fungicides amphotericin B and itraconazole with two replications. To determine the optimum fungicide concentration, four concentrations (0, 1, 10 and 20 μmol) were tested for each fungicide. Because 20 μmol of either fungicide had inhibited spore germination during the 7-day experimental period, this concentration was removed for the analysis of variance of the germ tube extension rate. The development of *A. niger* was sampled 7, 12, 17, 22, 48, 72, 96 and 168 h (or 7 days) after the initiation of the experiment according to Nesci *et al.* (2003). At each sampling time, spore germination rate, spore germ tube length, and later colony diameter were recorded. The germ tube extension rate, the time before spore germination, and fungal growth for each treatment was calculated. The data were analysed using PROC MIXED procedure of the SAS software (SAS Institute 2000) to ensure the correct error terms were used in the analysis. The mean values were separated using the Fisher's protected LSD test ($\alpha = 0{\cdot}05$).

RESULTS

Effect of a_w and fungicides on spore germination

Although either three-way interactions (a_w by fungicide by time), or any of the three two-way interactions did not significantly affect spore germination (P-values >0·05). The spore germination rate was affected significantly by a_w levels ($F = 13{\cdot}84$, d.f. = 3, 1466, $P < 0{\cdot}0001$), fungicides ($F = 4{\cdot}76$, d.f. = 1, 1466, $P < 0{\cdot}0293$), and sampling times ($F = 373{\cdot}16$, d.f. = 7, 1277, $P < 0{\cdot}0001$).

The data also demonstrated that fungicide concentration significantly inhibited spore germination rate (Fig. 1a–d, Table 1). For the standard water activity level ($a_w = 0{\cdot}994$), the lowest concentration (1 μmol) of either fungicide delayed 10% of spore germination until the 48-h sampling (Fig. 1b,d), whereas the controls reached a 10% of spore germination rate at the 7-h sampling. The lag phase observed for spore germination at the 1 μmol fungicide concentration would be an important factor to be used in the diets that are frequently changed because of the required high a_w level. In addition, the lower a_w levels ($a_w < 0{\cdot}994$) were found to further delay spore germination until the later sampling times of the 7-day experimental period (Table 1). Thus, to prevent *A. niger* spore germination in the semisolid insect diets it would be critical to determine the lowest a_w level in the diet that would not compromise insect development.

The lag phase for spore germination was affected significantly by a_w levels ($F = 73{\cdot}16$, d.f. = 3, 154, $P < 0{\cdot}0001$) and two fungicides ($F = 52{\cdot}15$, d.f. = 1, 154, $P < 0{\cdot}0001$) (Table 1). Among the controls for the fungicide treatment, the cultural medium with a higher a_w level showed a shorter lag phase for spore germination (Table 1). One μmol itraconazole was more effective than amphotericin B because a longer lag phase for spore germination at $a_w = 0{\cdot}961$ or 0·921 (Table 1) and less colony growth at all a_w levels (Fig. 2b,d).

Germ tube extension rate of the spores was only affected by water activity ($F = 14{\cdot}87$, d.f. = 3, 56, $P < 0{\cdot}0001$) (Table 1) and fungicide concentration ($F = 37{\cdot}93$, d.f. = 3, 56, $P < 0{\cdot}0001$). Among the two-way interactions, the germ tube extension rate was affected by a_w level by fungicide concentration interaction ($F = 3{\cdot}34$, d.f. = 2, 56, $P = 0{\cdot}0161$), but not by either two-way (a_w level by fungicide) or the three-way (a_w level by fungicide-by-fungicide concentration) interactions (P-values >0·05).

Effects of a_w and fungicides on colony growth

Colony growth of *A. niger* measured by colony diameter on the SDA medium was not affected by either three-way interactions (a_w by fungicide by time), fungicide-by-time, or a_w-by-fungicide interaction (P-values >0·05). The colony diameter was, however, significantly affected by a_w-by-time interaction ($F = 3{\cdot}94$, d.f. = 21, 1471, $P = 0{\cdot}0001$), a_w ($F = 27{\cdot}39$, d.f. = 3, 1471, $P < 0{\cdot}0001$), and sampling time ($F = 39{\cdot}16$, d.f. = 7, 1471, $P < 0{\cdot}0001$). The colony growth (Fig. 2a–d) corresponded to the spore germination data shown in Table 1. Although 1 μmol of both fungicides did suppress *A. niger* colony growth (Fig. 2b,d) when compared

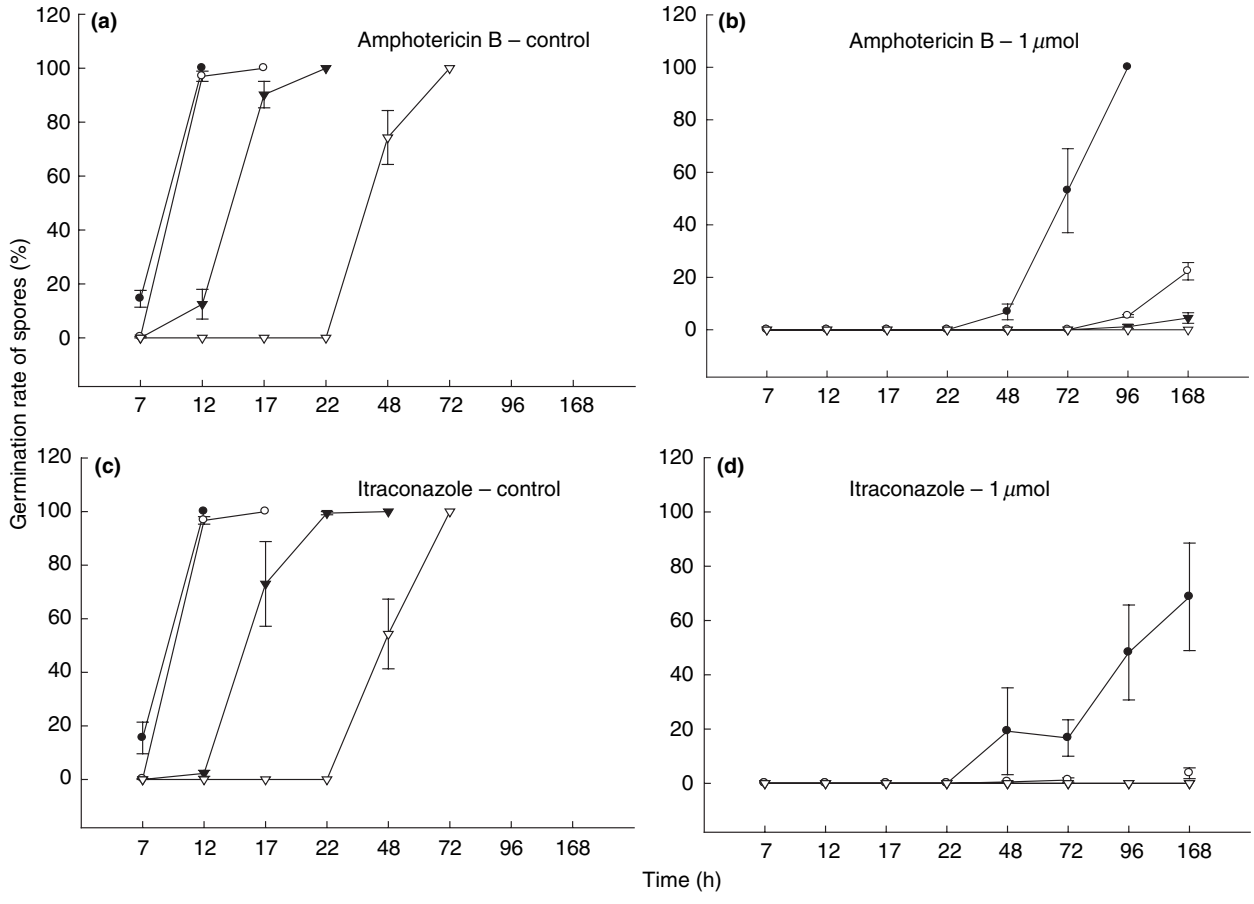

Fig. 1 Influence of four a_w levels and two fungicides on *Aspergillus niger* spore germination rate are shown for the 168 h experimental period ($n = 600$). Panels a and b are the control and 1 μmol of amphotericin B, respectively, whereas panels c and d are the control and 1 μmol of itraconazole respectively. The four lines within each graph are the four a_w levels [i.e. 0·994 (●), 0·961 (○), 0·921 (▼) and 0·859 (▽)]. The error bars are SEM

Table 1 Effect of fungicides on spore germination of *Aspergillus niger* at different a_w levels

	a_w levels							
	0·994		0·961		0·921		0·859	
μmol	AMB	ITR	AMB	ITR	AMB	ITR	AMB	ITR
	Time needed for 10% spore germination within 7 days ($n = 30$)							
0	7	7	12	12	16·3 ± 0·42	16·3 ± 0·40	48 ± 0·42	48
1	48	39·3 ± 5·3	56 ± 5·1	120 ± 7·7	88 ± 5·5	>168*	>168	>168
10	104 ± 17·2	160 ± 5·1	148 ± 15·7	>168	>168	>168	>168	>168
20	>168	>168	>168	>168	>168	>168	>168	>168
	Extension rate (μm h^{-1}) of spore germ tube ($n = 30$)							
0	14·6 ± 3·1	15·7 ± 2·5	21·2 ± 2·28	1·6 ± 2·37	9·5 ± 1·4	10·7 ± 2·4	3·5 ± 0·6	2·5 ± 0·3
1	4·1 ± 1·4	0·4 ± 0·1	5·8 ± 2·3	0·5 ± 0·2	4·5 ± 1·9	–	1·8 ± 0·8	–
10	1·1 ± 0·6	–	1·13	–	–	–	–	–
20	–	–	–	–	–	–	–	–

AMB, amphotericin B; ITR, itraconazole; –, no germ tube for measurement.
*>168 = 10% of spore germination was not achieved in 7 days (168 h).

 Letters in Applied Microbiology, **41,** 428–433, doi:10.1111/j.1472-765X.2005.01761.x

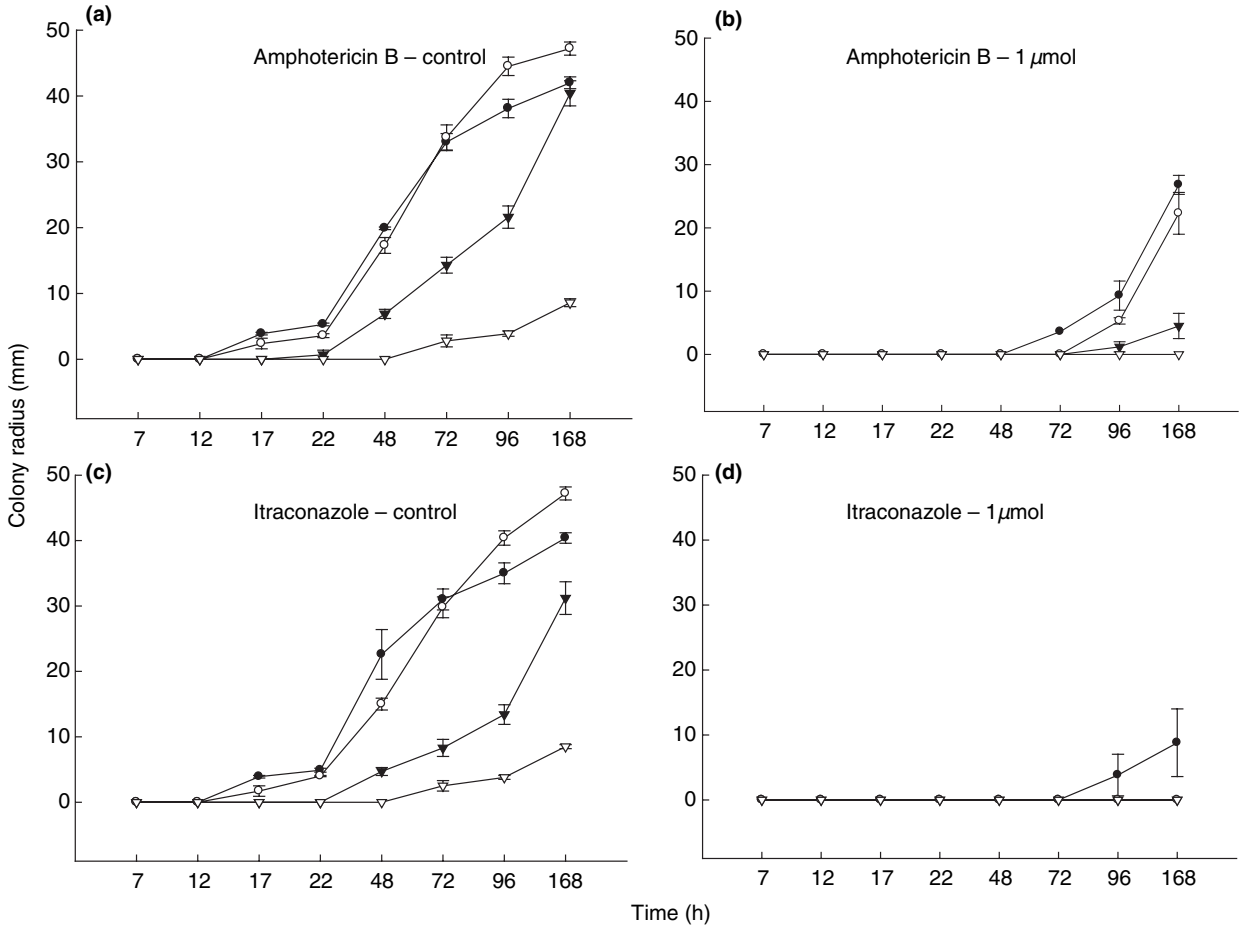

Fig. 2 Growth rate of *Aspergillus niger* colony measured by the diameter of a colony on the SDA medium ($n = 6$), control (a), 1 μmol of amphotericin B (b), control (c) and 1 μmol of itraconazole (d). The four lines within each graph are four a_w levels [i.e. 0·994 (●), 0·961 (○), 0·921 (▼) and 0·859 (▽)]. The error bars are SEM

with the controls (Fig. 2a,c), the effect of itraconazole is greater than amphotericin B on the colony growth. The impact of low a_w on the mycelial growth measured by the colony diameter was proportional with a_w values in the treatment of 1 μmol amphotericin B (Fig. 2b), whereas no mycelial colony was formed in any of the itraconazole treatments, except for the control (Fig. 2d).

Discussion

The present study demonstrated that the combination of low a_w ($a_w < 0{\cdot}96$) and low level (1 μmol) of itraconazole could delay *A. niger* spore germination for at least 5 days and prevent colony formation in 7 days, which could potentially be used in preventing *A. niger* contamination in artificial diets for insect mass rearing. Although four a_w levels (0·994, 0·961, 0·921 and 0·859) were used here, an a_w value of 0·961 is still the optimum for *A. niger* development as demonstrated by the colony diameter (Fig. 2). The lag phase of 5 days observed at $a_w = 0{\cdot}961$ with 1 μmol of itraconazole for spore germination (Table 1) and no colony formation in 7 days (Fig. 2d) would be valuable for developing insect diets that require relatively high a_w level. The finding supported previous reports that 0·97 was an optimum value for *A. niger* growth (Ayerst 1969; Marin *et al.* 1998; Parra and Magan 2004). Although similar studies have been conducted previously, they were all from food science literature in suppressing *Aspergillus* spp. contamination during grain (Marin *et al.* 1998; Paranagama *et al.* 2003) or cooked food (Hocking 1997; Jay 2000; Nesci *et al.* 2003; Parra *et al.* 2004) storage. The present study is one of the first attempts to assess the combined effect of fungicides and a_w on preventing *A. niger* contamination in a common cultural medium (SDA), which has similar physical texture with several artificial diets for the mass rearing of insects for commercial and research uses.

When the two fungicides were compared, amphotericin B was less effective than itraconazole at 1 μmol in suppressing

mycelial growth. The finding was in agreement with previous report by Provine and Hadley (2000). They reported that itraconazole was more effective than amphotericin B in suppressing *Aspergillus* spp. and other filamentous fungi in a semisolid medium.

The data on spore germination rate, lag phase of spore germination, germ tube extension rate, and colony growth have demonstrated that the combination of itraconazole with reduced a_w could effectively prevent *A. niger* contamination on the SDA cultural media for at least 7 days. The best choice for controlling *A. niger* collected from insect-rearing facilities is the combination of 1 μmol of itraconazole and $a_w < 0{\cdot}961$. The results from this study provide critical baseline information for further examinations to optimize a_w and fungicide levels in insect artificial diets. It would be critical to determine the optimum a_w and fungicide levels in an insect diet that would not compromise normal insect development.

ACKNOWLEDGEMENTS

We greatly appreciate the outstanding technical assistance by D.G. Smith (Biological Control and Mass Rearing Research Unit, USDA-ARS, Mississippi State, MS). We would also like to express our gratitude to D. Boykin (USDA-ARS, Stoneville, MS) for her valuable advice on statistical analysis. We also thank H. Abbas (USDA-ARS, Stoneville, MS), J. Alverson (USDA-ARS, Pullman, WA), and F. Davis (Department of Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS) for their critical review of the earlier draft of this manuscript.

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